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## Minireview

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## Pseudomonas Exotoxin: Chimeric Toxins

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Toxins are elaborated by microorganisms presumably to give them a selective growth advantage in certain environments. Thus, toxins can protect bacteria from hostile environments and help them to secure nutrients. Seeds of plants also contain toxins where they may have a protective function and aid in seed dispersal. Some toxins act at the surface of target cells; others must be internalized and enter the cytosol to kill target cells. Because toxins can readily kill human cells, they are being investigated as possible therapeutic agents against several diseases. This is done by altering the structure of the toxin so that it will bind preferentially to disease-causing cells instead of binding to normal cells. Initially targeting of toxins was done by chemically attaching a whole toxin or a fragment of a toxin to an antibody to produce an immunotoxin; the antibody is directed at an antigen uniquely present on the surface of a target cell (1-3). Several types of cancer cells have been found to contain unusually large numbers of growth factor receptors; therefore, toxins have also been chemically attached to growth factors or to anti-receptor antibodies which target them to receptor-bearing cells (4-13).

Toxin molecules that act within the cell contain at least three functional domains (14). One domain directs the toxin to the cell surface by binding to a component on the cell membrane. A second domain contains the enzyme activity that inactivates an essential cellular function and results in cell death. A third domain helps the cytotoxic domain translocate across the cell membrane in order to reach the cytosol where the target of the toxic domain is located.

The potential of using toxins as therapeutic agents has stimulated research into the structure and activity of these molecules. One very important advance was the solution of the three-dimensional structure of *Pseudomonas* exotoxin (15). Guided by this structure it has been possible to determine the location of the various functional regions of *Pseudomonas* exotoxin (16) and to use this information to construct novel forms of the toxin that are selectively cytotoxic for cells with specific target molecules on their surface. This review will focus on the structure activity relationships of *Pseudomonas* exotoxin and compare some of their properties to those of diphtheria toxin, ricin, and other toxins where appropriate.

Both diphtheria toxin and Pseudomonas exotoxin kill cells by catalyzing the transfer of the ADP moiety derived from NAD to a modified histidine residue, termed diphthamide, present in elongation factor 2 (EF-2)<sup>1</sup> (17-19). Because both toxins modify the same residue, cell mutants that are resistant to one toxin are often cross-resistant to the other. The toxins also have similar molecular masses, Pseudomonas exotoxin is 66 kDa and diphtheria toxin is 60 kDa. Therefore, one might expect there to be considerable homology in the DNA or amino acid sequences of the two toxins, but there is not. The two molecules show a small amount of sequence homology, and this occurs primarily in the region known to bind NAD and catalyze the ADP-ribosylation reaction (20, 21).

The steps in toxin action are shown schematically in Fig. 1. In this model we have incorporated data from both diphtheria toxin and *Pseudomonas* exotoxin. The first step is binding of the toxin to a cell surface receptor; to date the biochemical nature of the receptor for

The abbreviations used are: EF-2, clongation factor 2; DT, diphtheria toxin; PE40, portion of *Pseudomonas* exotoxin containing amino acids 253-613 (PE40 lacks the binding domain);  $TGF\alpha$ , transforming growth factor  $\alpha$ ; EGF, epidermal growth factor; IL, interleukin

either toxin is still not clear. Next the toxins enter coated pits from which they are transferred to endocytic vesicles (22-24). These vesicles contain a proton pump which rapidly acidifies the vesicles shortly after their formation. The low pH alters the toxin structure exposing hydrophobic residues which probably help the toxin to insert into the membrane (25-29). Agents that raise intravesicular pH such as chloroquine or ammonium chloride block the action of these toxins (22, 30, 31), whereas they potentiate the action of plant toxins such as ricin and abrin indicating that the plant toxins have a different site or mechanism of entry into the cytosol (32). Eventually the toxin gains access to the cytosol probably as an enzymatically active fragment and inactivates EF-2. In the case of Pseudomonas exotoxin, the nature of the fragment is not yet known. Olsnes and colleagues (33) have identified several fragments of diphtheria toxin that become inserted into the membrane in an acid pH-dependent reaction that follows the binding step. It appears that the diphtheria toxin fragment present in the cytosol is the 20-kDa A chain (33).

The three-dimensional structure of *Pseudomonas* exotoxin shows it to be made up of three major domains (15) with domain I subdivided into a large domain (Ia) and a small domain (Ib). These two portions of domain I are separated in the DNA sequence but lie close together in the three-dimensional structure of the protein (see Fig. 2).

To ascertain the function of each domain, DNA fragments containing or lacking the domain(s) of interest were isolated using standard recombinant DNA techniques and expressed into protein in Escherichia coli (16). DNA fragments encoding different portions of the Pseudomonas exotoxin gene were cloned into an expression plasmid so that large amounts of recombinant toxin could be made and purified. A summary of some of the proteins made is shown in Fig. 3. Deletion of domain Ia (amino acids 1-253) resulted in a M, 40,000 molecule, PE40, which has low toxicity on cultured cells but full ADP-ribosylation activity. The low toxicity, which could have been due to either diminished cell binding or diminished translocation activity, was shown to be due to low binding based on the following data: (i) a protein composed of only domain I was prepared and was shown to bind to cells and block the cytotoxic effect of Pseudomonas exotoxin in a concentration-dependent manner (16); (ii) PE40 (domains II, Ib, III) which has very little cytotoxicity by itself was conjugated to several different antibodies or growth factors (see below) which bound to and killed different types of target cells (34).

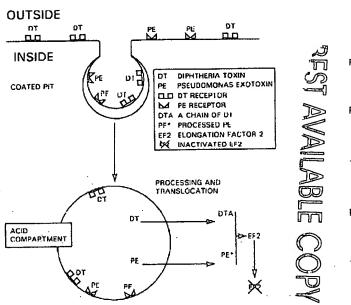
Domain III was shown to contain the ADP-ribosylating activity of Pseudomonas exotoxin (16). Expression of a plasmid-encoding domain III produced a protein of M, 24,000 which had ADP-ribosylating activity equivalent to native Pseudomonas exotoxin, whereas expression of Pseudomonas exotoxin genes encoding only domains I or II produced proteins with no enzymatic activity.

Finally, a plasmid encoding a protein with a deletion ( $\Delta 253-308$ ) of half of domain II (amino acids 253-364) was constructed and shown to produce a protein that had both cell-binding and ADP-ribosylating activities but could not kill cells (16). By deduction, since the other *Pseudomonas* exotoxin functions were present in this mutant, domain II must be needed for translocation.

#### Mutations in Domain Ia

When Pseudomonas exotoxin was treated with reagents that react with free amino groups such as 2-iminothiolane, the modified molecule displayed decreased cytotoxic activity without decreased ADP-ribosylating activity (35, 36); this indicated that the function of domain III was intact. Because the modified Pseudomonas exotoxin molecule could be attached to antibodies or to epidermal growth factor to produce an active cell-killing reagent, domain II also had to be functional. Therefore, it seems very likely that iminothiolane modified a free amino group present in domain la. There are 12 lysines in domain Ia that could have reacted with iminothiolane besides the amino group of the NH<sub>2</sub> terminus, and it seemed likely that modification of one or more of the amino groups in this domain was responsible for the decrease in cytotoxicity. Accordingly, each lysine in domain I was changed to a glutamate residue by site-directed mutagenesis (37). Most of the mutations had little or no effect on the

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Fig. 1. Entry of *Pseudomonas* exotoxin and diphtheria toxin into animal cells.

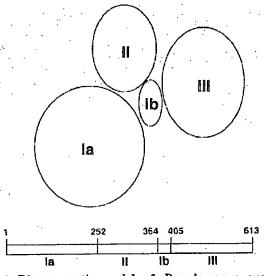


Fig. 2. Diagrammatic model of *Pseudomonas* exotoxin showing domains Ia, II, Ib, and III (15).

cytotoxic activity of *Pseudomonas* exotoxin, except for a mutation at position 57. When Lys-57 was changed to Glu, there was a 100-fold loss of cytotoxic activity, and this loss of cytotoxic activity was due to decreased binding of the mutant toxin to the surface of the cells (37). Lysine 57 has been shown to lie on the surface of the *Pseudomonas* exotoxin molecule consistent with a role in receptor binding (15, 37).

#### Mutations in Domain II

Domain II of Pseudomonas exotoxin is composed of 112 amino acids (amino acids 253-364) and contains six helices. Two of these helices, B and E, consist of 21 amino acids each, a length sufficient to span a membrane. The other four helices are smaller. Insertion of the helices into or across a membrane could be an important part of the translocation process.

To try and identify crucial sequences in this domain, a series of deletion mutunts have been made (38). All of these mutants have greatly decreased cytotoxic activity yet retain normal ADP-ribosylating activity. To ensure that the mutations in domain II did not affect the binding of mutant Pseudomonas exotoxin molecules to target cells, domain I of Pseudomonas exotoxin was also deleted and replaced with transforming growth factor  $\alpha^*(TGF\alpha)$  to create a chimeric toxin hound to cells with epidermal growth factor (EGF) receptors (Fig. 3).

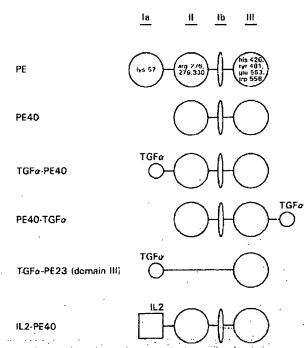


Fig. 3. Domains of Pseudomönas exotoxin (PE), PE40, and Pseudomonas exotoxin-gene fusions. Key amino acids for Pseudomonas exotoxin function are located in various domains; PE40 has translocating and ADP-ribosylating activity but no cell binding; TGFα-PE40 is more active than PE40-TGFα; TGFα-PE40 does not kill cells since it has no translocating (domain II) activity; II.2-PE40 binds to and kills cells expressing II.2 receptors.

The function of domain II has also been studied by the creation of point mutations. Initially, positively charged amino acids were investigated for their role in translocation. Domain II has 12 arginine residues and no lysines. Therefore, each of the arginine residues was changed by mutation (39). The most dramatic decrease in cytotoxicity was brought about by mutations in Arg-276 and Arg-279 (Table I). Changing Arg-276 to Gly produced a molecule with a complete loss of cytotoxic activity and changing Arg-279 to Gly caused a 400-fold fall in cytotoxicity (39). Arginine 276 was also changed to Glu, His, Lys, and Gln, and all these mutants had extremely low cytotoxicity. Arginines 276 and 279 lie in a loop on the surface of domain II that connects the A and B helix. In that location Arg-276 and -279 should be able to interact with components of the membrane through which Pseudomonas exotoxin must translocate. The fact that Arg-276 cannot be replaced with amino acids of similar size or charge suggests that it participates in a very specific interaction with a cellular component. To demonstrate the existence of such an interaction, competition experiments using wild type and mutant molecules were carried out. These indicated that Arg-276 was necessary for a ratelimiting (saturable) reaction that occurred within an intracellular compartment, and this interaction was probably involved in the translocation reaction (39). This rate-limiting step could involve the binding of Pseudomonas exotoxin to a translocating protein or be involved in the processing of Pseudomonas exotoxin as part of the translocation process.

#### Mutations in Domain III

The ADP-ribosylation activity of Pseudomonas exotoxin is contained within structural domain III. Pseudomonas exotoxin has NAD glycohydrolase and ADP-ribose transferase activity, and it interacts with elongation factor 2. The functional boundaries for these activities are now being defined. The data of Allured et al. (15) indicated that structurally domain III begins at amino acid 405. To ascertain in a functional assay (ADP-ribosylation) the location of the NH2-terminal boundary of domain III, a set of deletion mutants was created which removed all of domains I and II and encroached to various degrees on domain III. Removal of all the amino acids prior to residue 401 resulted in a protein with full ADP-ribosylation activity (38). But removal of only four more amino acids decreased activity by 50%, and a protein which began at position 408 had almost a complete loss of the ADP-ribosylation activity (38). This result clearly shows that

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#### TABLE I

Amino acids important for Pseudomonas exotoxia function

To determine the location of key amino acids that are necessary for toxin activity various investigators have made single amino acid changes in Pseudomonas exotoxin. These mutant forms were assayed either for Pseudomonas exotoxin binding, translocation to the cytoplasm, or ADP-ribosylation activity. The functional deficits caused by these changes are summarized in this table.

Amino acid(s)		Domain		D. 1	5.4
location	Binding	Translocation	ADP-ribosylation	Remarks	Refs.
Lys-57	+			Glu-57, reduced cell killing	37
His-246-Gly-252	?	?		More toxic than PE40; reason not clear	37
Arg-276	'n	. +		Reduced cell killing by 1000-fold when Arg is changed to other amino acid	39
Arg-279		+		Reduced cell killing by 500-fold when Arg is changed to other amino acid	39
Arg-330	T	+		Reduced cell killing by 30-fold	39
His-426			<b>+</b>	Deletion of 426 has no ADP-ribosyla- tion activity	43
Glu-553			+	Asp-653 has low ADP-ribosylation activity	41
			+	Deletion at 553 has no ADP ribosylu- tion activity	42
Trp-558			+	May be important for ADP-ribosyla- tion activity	43
Tyr-481			+	Inhibits ADP-ribosylation when iodi- nated or mutated	21, 44

TABLE II Cytotoxic activity of Pseudomonas exotoxin and chimeric toxins on cell lines with different receptors

Cell line	Receptor	Number	TGFa-PE40	1D <sub>80</sub>					
Oen me				IL2-PE40	fL6-PE40	IL4-PE40	CD4-PE40		
				ng/ml					
A431	EGF	$2 \times 10^{6}$	0.1	>1,000	>1,000	>1,000	ND"		
KB	EGF	$2 \times 10^5$	0.2	>1,000	>1,000	>1,000	ND		
HUT-102	II.2	50,000	>1,000	2	>1,000	>1,000	ND		
U266	IL6	12,000	>1,000	>1,000	10	ND	ND		
CT.4R	IL4	15,000	ŇD	200	>1,000	17	ND		
HIV-H9	gp120	Large	ND	ND	ND	ND	<0.1		

<sup>&</sup>quot; ND, not determined.

functionally domain III begins around amino acid 400 and very little of the amino end of domain III can be removed without loss of enzymatic activity. Deletion mapping of the COOH end of domain III has not yet been performed.

Within domain III, various key amino acids have been identified (Table I). Photolabeling has shown the importance of Glu-553 for binding NAD (40). Changing this residue to Asp reduces ADPribosylation by at least 100-fold (41), and deleting it eliminates activity completely (42). Two other amino acids, His-426 (43) and Trp-558 (43), not directly involved in NAD binding also appear necessary for ADP-ribosylation. Wozniak et al. (43) have suggested that the ADP-ribosylating activity of a variety of bacterial toxins requires the presence of three specific amino acids; these are His, Glu, and Trp arranged with a 125- or 126-amino acid separation between His and Glu and a 3- or 4-amino acid separation between Glu and Trp. It has been known for some time that iodination of Pseudomonas exotoxin by chloramine-T methods<sup>2</sup> can cause loss of ADP-rihosylating activity. The iodination of tyrosine 481 apparently introduces an atom of iodine that abolishes ADP-ribosylation activity (21). Changing Tyr-481 to Phe causes reduction in ADP ribosyltransferase activity but not in NAD binding or NAD glycohydrolase activity (44). Therefore, this amino acid may be involved in the interaction of domain III with EF-2 (44).

#### Chimeric Toxin Molecules

Chimeric toxin molecules have been created by deleting a portion

<sup>2</sup> D. FitzGerald, unpublished data.

of the toxin gene encoding cell binding information and replacing it with DNA coding for peptide hormones, growth factors, or other recognition proteins. This approach has been used to make chimeric proteins with both Pseudomonas exotoxin and diphtheria toxin (45-50). No gene fusions with cytotoxic portions of ricin have yet been reported.

As indicated in Fig. 3, several growth factors and lymphokines have been used to replace domain la of Pseudomonas exotoxin in order to direct the toxin to cells hearing different types of receptors. To do this, a cDNA encoding a specific growth factor or lymphokine has been ligated to a DNA fragment encoding domains II, Ib, and III of Pseudomonas exotoxin. A list of the chimeric molecules constructed in this manner and their activity on cell lines with different receptors is shown in Table II and includes molecules that bind to the EGF receptor as well as to the interleukin 2 (IL2), interleukin 4, and interleukin 6 receptors (45-48). HIV, the virus responsible for AIDS, infects T cells by binding to a cell surface protein termed CD4. A chimeric toxin that kills HIV-infected cells was created by fusing a cDNA encoding amino acids 1-178 of CD4 to DNA for Pseudomonas exotoxin domains II, Ib, and III (51). This chimeric protein, CD4-PE40, binds to a glycoprotein (gp120) that is found on the surface of HIV-infected cells and kills these cells (5)).

Two types of chimeric toxins containing  $TGF\alpha$  have been constructed. Initially TGFa was placed at the COOH end of PE40 and connected to it by a short linking peptide (45). In the other construction TGFa was placed at the NH2 terminus of PE40 with no linking peptide (38). Both forms of the molecule had full ADP-ribosylating activity and were cytotoxic to cells that contain EGF receptors, but 15160

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TGFa-PE40 was about 10-fold more active than PE40-TGFa. This difference in activity could be due to differences in the binding of  $TGF\alpha$  to the EGF receptor or in some step in the translocation process. We have also constructed both IL2-PE40 and PE40-IL2 and found only IL2-PE40 to be cytotoxic.3 Together these results suggest that Pseudomonas exotoxin-related chimeric molecules with the ligand at the COOH terminus probably translocate poorly.

Making chimeric toxins by chemical linkage has been described for several combinations of toxins and cell-binding proteins. For instance, EGF has been linked to Pseudomonas exotoxin (5), ricin A chain, and diphtheria toxin A chain (4, 52). With the advent of gene fusions, however, chemical linkage has become the second choice for making Pseudomonas exotoxin and diphtheria toxin-related chimeras with small peptides although it is still used for antibodies. This is not the case for ricin A chain chimeras. Recently, soluble CD4 was linked to ricin A chain by a disulfide linkage (53). It should be noted that cytotoxic ricin A chain conjugates made either by chemical thioether linkage or by peptide linkage in gene fusions have not been reported and, if made, would be expected to be inactive, because in native ricin the carboxyl end of the A chain is disulfide-linked to the NH2terminal end of the B chain: A disulfide arrangement is probably needed to make an active ricin A chain chimera.

One obvious use for chimeric toxins is the treatment of human and animal diseases. Diseased cells or disease-causing cells serve as targets and can be cradicated by the injection of chimeric toxins. For example, prolongation of the survival of cardiac allografts in mice has been reported by testing mice with II.2-PE40 or IL2-DT at the time of engraftment (54, 55). By killing IL2 receptor-bearing cells, the chimeric toxin in some way prevents the emergence of cylotoxic T cells. In other studies, IL2-PE40 was shown to suppress or prevent an experimental form of arthritis in rats which is due to the production of activated T cells (56). Chimeric toxins have also been used as probes for receptor studies. IL2-PE40 will kill cells bearing either the p55 kDa subunit or the p75 kDa subunit, but quite large amounts are required (57). However, if both subunits are present, much lower concentrations of IL2-PE40 are required to kill cells, because receptors containing both receptor chains bind IL2 with higher affinity and are internalized more efficiently. The selection of receptordeficient cells has also been accomplished using chimeric toxins (58). Since toxin internalization is required to gain access to the cytoplasm, processes relating to receptor-mediated endocytosis can also be studied.

#### Summary

Pseudomonas exotoxin binds to and enters cells by receptor-mediated endocytosis. Within the cell it requires exposure to low pH to enable it to translocate to the cell cytoplasm where it inhibits protein synthesis by ADP-ribosylating elongation factor 2. The toxin has three main structural domains whose functions are: Ia, cell binding; II, translocation; and III, ADP-ribosylation. Key amino acids have been identified within each domain that are required for the function of the toxin.

Chimeric toxins were made originally by using chemical crosslinking reagents to couple Pseudomonas exotoxin (or other toxins) to cell-binding proteins. More recently, a variety of Pseudomonus exotoxin-related chimeric toxins have been made by gene fusion technology. These chimeric toxins may be useful clinically for treating various diseases and experimentally for understanding receptor function.

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